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14. ABSTRACT

A major consequence of spinal cord injury (SCI) is the development of a glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory chondroitin sulfate proteoglycans (CSPGs) are elevated in the glial scar and are a major deterrent to successful regeneration. In the present study, we have demonstrated that injured astrocytes produce a wide variety of inhibitory CSPGs. To develop a more efficient method to accomplish CSPG degradation (than the bacterial enzyme chondroitinase), we are addressing a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. We have begun a two-prong approach, employing studies both in vitro, and in vivo. We have produced recombinant ADAMTS-4 protein for experiments in-vitro, or for injection in vivo, alone and in combination with chondroitinase. Thus far, we have focused mainly on the development of critical reagents and methods, and have tested these in proof of principle assays. In parallel, we have begun to develop a reproducible SCI model and are developing behavioral assessments to validate the success of aggrecanase and chondroitinase treatments. The significance of these stages is that they will lead to an efficient means by which to attenuate axonal inhibition, and thereby promote plasticity and regeneration of adult neurons following SCI.

15. SUBJECT TERMS

astrocytic scar; CSPGs; recombinant ADAMTS-4; dorsal hemisection model; pellet retrieval task

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Table of Contents

Page

Introduction

Subject. Spinal cord injury (SCI) is a devastating condition affecting as many as 306,000 individuals in the US alone *(http://www.brainandspinalcord.org/spinal-cord-injury/statistics.html)*. Beyond US soil, SCI is an all-too-common result of military combat – all with an enormous emotional, social, and financial cost to society. Despite much needed attention over the past few decades and some significant advances, the cellular and molecular mechanisms leading to SCI are not yet clear. *Purpose:* A major consequence of SCI is the development of an astrocytic glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory **chondroitin sulfate proteoglycans (CSPGs)** are elevated in the glial scar and are a major deterrent to successful regeneration. To develop a more efficient method to accomplish CSPG degradation (than the current use of the bacterial enzyme chondroitinase), we are studying a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. *Scope:* We are using a two prong approach – employing studies both *in vitro*, and *in vivo*. We are isolating, purifying and testing aggrecanase on astrocytes in tissue culture; and we will test aggrecanase lenti-viral constructs in an injury model *in vivo*, alone and in combination with chondroitinase, to ameliorate CSPG-induced inhibition. Thus far, we have focused mainly on the experimentation *in vitro* to develop reagents and for proof of principle, but we have also begun to develop our *in vivo* model in parallel with the work *in vitro* for future application.

The following is a Progress Report for CDMRP grant SC090248, entitled "A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration". Each point of progress is addressed in relationship to the Statement of Work provided in the original application.

Statement of Work

Overarching goal: *To successfully degrade aggrecan and related CSPGs using naturally occurring ADAMTS-4, alone and in combination with the current "gold standard" (chondroitinase) to attenuate axonal inhibition, and promote plasticity and regeneration of adult neurons of the CNS.*

Task 1. We will determine if aggrecanase and chondroitinase-mediated degradation of CSPGs produced by primary rat cortical astrocytes will foster neurite outgrowth *in vitro*.

1a. We will confirm that primary astrocytes upregulate CSPGs *in vitro* in response to injury (experimentally induced by trauma and/or administration of TGF-beta (Smith and Strunz, 2006). We will also catalogue the specific PGs upregulated and their time course (months 1-4).

Neonatal rat cortical astrocyte cultures were prepared and expanded to 16 T-75 flasks grown to near confluency. The cultures were incubated for 3 days with 2.5 ng/ml TGF-beta in 5 ml of medium. Medium was collected from 16 flasks, and guanidine HCl was added to a final concentration of 4M. The cell layers were washed with PBS, and were lysed in 0.05M sodium acetate, pH 5.8, containing 4M guanidine HCl, 0.5% CHAPS and protease inhibitors. An additional two flasks of cells were lysed, and used for the preparation of total cellular RNA, for quantitative real time PCR analysis of proteoglycan mRNA. Samples in guanidine buffer were used for purification and characterization of astrocyte proteoglycans. Briefly, samples were chromatographed on Sephadex G-50 equilibrated with 0.05M sodium acetate, pH 6.0 containing 0.5% CHAPS, 0.15 M sodium chloride, and 8M urea. The void

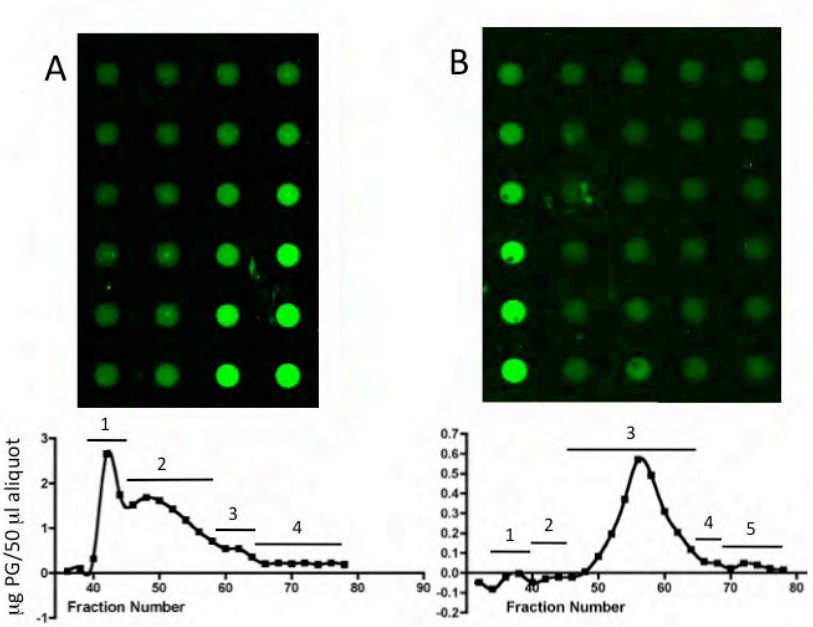

Figure 1. Analysis of proteoglycans produced by activated rat cortical astrocytes. Proteoglycans were purified by size exclusion and ion exchange chromatography. DEAE chromatographic profiles are shown for astrocyte cell layer (A) and medium (B) eluted with a 0.15 to 1.0 M NaCl gradient. Fractions were pooled, concentrated and characterized by dot blot analysis using specific antibodies to different proteoglycans. The dot blot analysis above the chomatogram shows reactivity of serial dilutions of pooled fractions with an antibody to the G1 domain of aggrecan.

fractions were applied to a column of DEAE Sephacel equilibrated in the same buffer. The column was washed, and proteoglycans were eluted using a 0.15 to 1.0 M sodium chloride gradient **(Figure 1).** The purification protocol was first tested using conditioned medium from rat chondrosarcoma cells, which secrete abundant aggrecan into the medium.

Chromatography of medium and cell layers from astrocyte cultures each revealed several peaks that were pooled, buffer-exchanged to remove urea, and concentrated (Figure X). Dot blot analyses of pooled fractions using antibodies specific for different CSPGs were performed. Both medium and lysate contained the CSPGs: phosphacan, NG2, versican, neurocan, and aggrecan. Decorin was abundant in the cell lysate, but virtually absent from the medium. Each of these pools is currently being analysed by SDS-PAGE with and without chondroitinase ABC and keratanase digestion to visualize core protein size and reactivity with specific antisera. Chromatography was also performed on HEK-293 produced proteoglycans (*see HEK293 explanation in 1b below*), which showed multiple peaks eluting at increasing NaCl concentrations from the DEAE column **(Figure 2).** Each peak was analyzed by dot blot assay with specific CSPG antisera. Aggrecan, neurocan, versican, phosphacan and NG2 were detected in each peak.

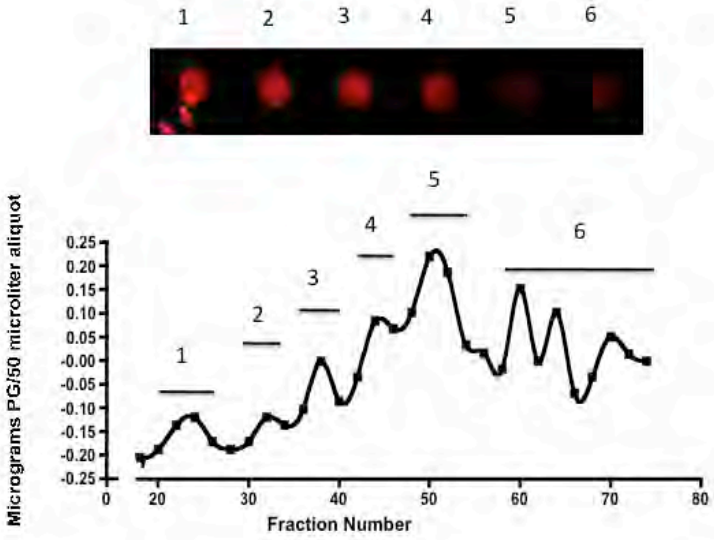

Figure 2. Analysis of proteoglycans secreted into culture medium by HEK293T cells following transfection with a FLAG-tagged aggrecan expression vector. DEAE chromatographic profile is shown, with fractions eluted over a 0.15 to 1.0 M NaCl gradient. Top panel shows dot blot analysis of pooled fractions using an antibody directed toward the G1 domain of aggrecan.

The multiple peaks are believed to be due to different degrees of sulfation of these proteoglycans. All of these proteoglycan pools will be further characterized for sulfation patterns using FACE or HPLC sulfated disaccharide analyses, and will be used for analysis of neurite outgrowth inhibitory activity using an assay developed in our laboratory and currently being optimized for this application.

1b. Using a lentiviral system, we will induce primary astrocytes to degrade CSPGs via aggrecanase, chondroitinase, or both. Degradation of CSPGs will be confirmed using an anti-C-4-S antibody (2-B-6), which indentifies CSPG stubs following chondroitinase cleavage, or by antibodies to neoepitopes that are generated when aggrecanase cleaves the CSPG protein core into specific fragments. (months 5-7).

Cloning of ADAMTS-4 expression constructs. We have generated three ADAMTS-4 expression constructs. The first plasmid (designated 701) contains the ADAMTS-4 coding sequence cloned into the vector pcDNA3.1/myc-his(-)A. Full sized (90 kDa pro-protein) ADAMTS-4 expressed from this plasmid, as well as the processed 68 kDa secreted form, can be detected with antibodies to ADAMTS-4, myc or His epitopes. The C-terminal His-tag enables purification of the protein on a nickel-chelating resin (i.e. ProBond). The second vector (designated 702) has an ADAMTS-4 insert lacking the N-terminal propeptide, with an N-terminal FLAG sequence adjacent to the first residue of the "processed" 68 kDa ADAMTS-4 sequence. This vector was constructed to determine if an N-terminal FLAG sequence would be more likely to persist in the expressed protein, since autocatalytic C-terminal proteolytic cleavage may remove a C-terminal FLAG sequence. A third construct (designated 703) places an ADAMTS-4 insert having a C-terminal FLAG sequence in the vector pcDNA3.1. The protein product can be affinity purified on a anti-FLAG affinity matrix, and can be detected on a Western blot using an anti-FLAG primary antibody. Each construct can be transiently or stably transfected into mammalian cells.

To produce recombinant ADAMTS-4 protein for experiments *in-vitro*, or for injection into rat spinal cord injury sites (for Task 2a), we have transiently transfected the 703 construct (having a C-terminal FLAG tag) into HEK293T cells for protein expression. This cell line was chosen because it is commonly used for high-level recombinant protein expression. In addition, we and others have found that the HEK293T cell line has characteristics of neuronal progenitor cells. We have found that this cell line produces proteoglycans characteristic of the CNS, including aggrecan, versican, decorin, neurocan, NG-2 and phosphacan. When transfected with the ADAMTS-4 expression plasmid, we found high levels of recombinant protein expression, and discovered that the cells themselves produce ADAMTS4 endogenously **(Figure 3).**

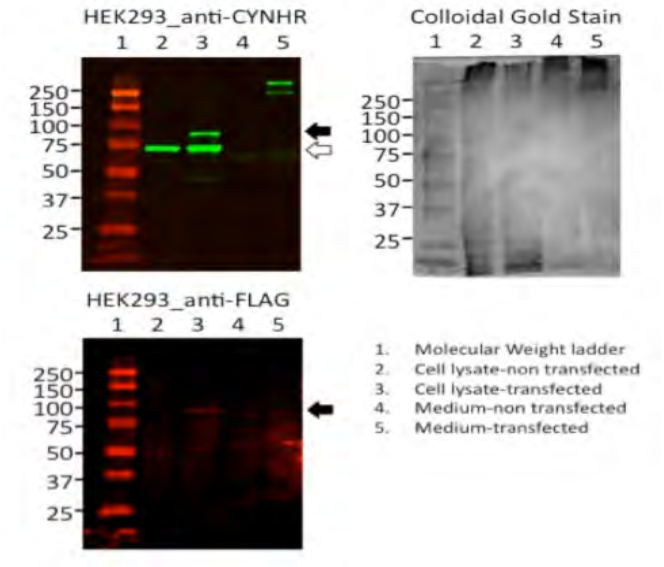

Figure 3. Expression of recombinant FLAG-ADAMTS4 in HEK293T cells. Samples analyzed include nontransfected (lane 2) and transfected (lane 3) cell lysate, and nontransfected (lane 4) and transfected (lane 5) medium. An antibody to ADAMTS4 revealed endogenous ADAMTS4 expressed by HEK293T cells (white arrow), and recombinant FLAG-tagged ADAMTS4 (as a 90 kDa proenzyme) seen only in transfected cell lysates (black arrow). Recombinant ADAMTS4 migrated near the top of the gel, suggesting association with proteoglycans. Colloidal gold staining of the immobilon membrane showed an apparent decrease in high molecular weight material (presumably proteoglycans) in the transfected lysate, and the appearance of a faster migrating band in the transfected medium.

The recombinant ADAMTS4 was found in both the cell layer and the medium. The enzyme in the medium, however, appeared to be in a complex with a high molecular weight material that is likely aggrecan or another CSPG. Examination of the western blot following colloidal gold staining, to reveal all of the protein in each lane, showed a reduction in very high molecular weight protein in the cell lysate and the medium from ADAMTS4-transfected cells. This suggested that aggrecan, or other CSPGs produced by the HEK293 cells were actually being degraded by the expressed protease. Subsequently, another western blot analysis of lysate and medium from the same experiment showed the presence of ADAMTS4-generated aggrecan fragments, using an antibody to a specific neoepitope (anti-NITEGE) found on aggrecanase-generated aggrecan fragments **(Figure 4).**

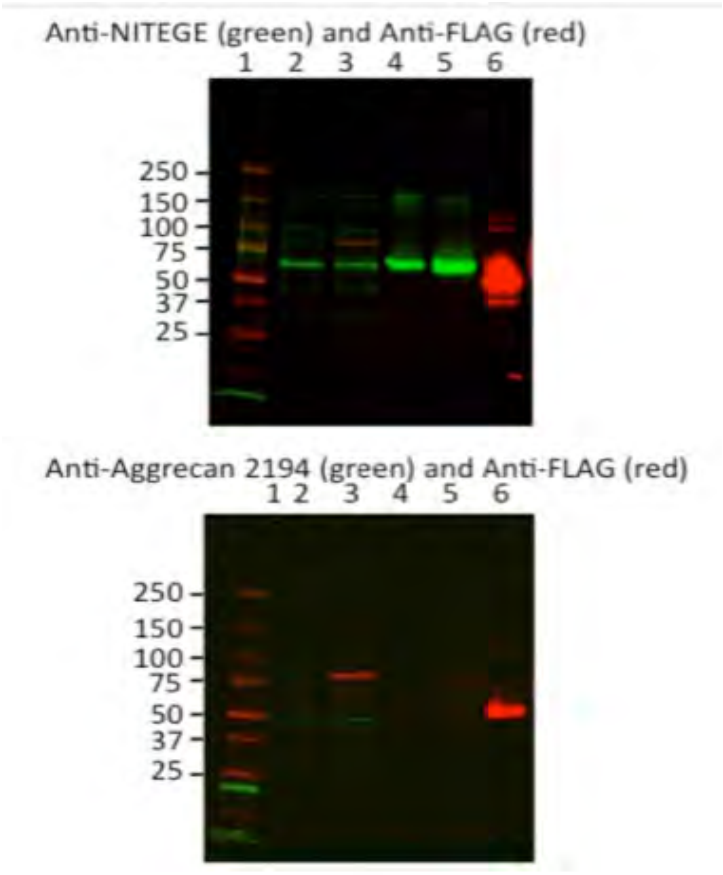

Figure 4. Evidence for aggrecan cleavage by recombinant ADAMTS4 expressed by HEK293T cells. Samples analyzed were the same as in Figure 3. Lane 6 is a FLAG-bacterial alkaline phosphatase control for the anti-FLAG antibody. Each blot was simultaneously reacted with anti-NITEGE and anti-FLAG (top figure) or anti-aggrecan and anti-FLAG (bottom figure). The full sized recombinant ADAMTS4 is expressed as a ~90 kDa proenzyme (red band, lane 3), and as the active 68 kDa enzyme in the medium (red band, lane 5). Top panel shows that an ADAMTS4-generated proteolytic fragment of aggrecan (NITEGE antibody) is more abundant in the medium of transfected cells (top panel, green band, lane 5). A band at the same position also reacts with anti G1 domain antibody (bottom panel, red band, lane 5).

This fragment was seen in non-transfected lysates, apparently due to the endogenous ADAMTS4 activity, but was significantly increased in the medium from transfected cells. We regard this result as proof of principle for our proposed future experiments, which will be to express ADAMTS4 in glial scar to reduce the content of neurite outgrowth-inhibitory CSPGs. We are currently working to purify recombinant ADAMTS4 from transfected HEK293 medium. Following transfection, HEK293 cells are being incubated in medium containing heparin, to antagonize the strong association of ADAMTS-4 with PGs in the cell layer and medium. The enzyme will then be purified using FLAG antibody-conjugated magnetic beads **(Figure 5).**

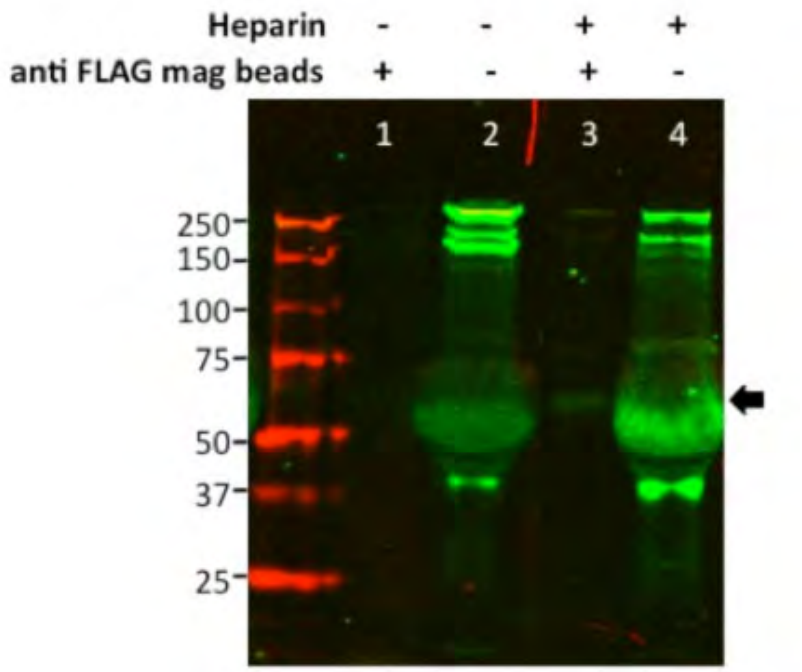

Figure 5. Purification of recombinant ADAMTS4 from transfected HEK293T cells. Anti-FLAG-conjugated magnetic beads were used to purify FLAG-ADAMTS4 from culture medium. Addition of heparin to the cultures prevented association of ADAMTS4 with proteoglycans (note reduced reactive material at top of gel in lane 4). Purified recombinant aggrecan migrated as a 68 kDa band (lane 3).

Preparation of pLVX-Tet-On Advanced, and pLVX-Tight-Pur-ADAMTS-4 vectors. We have developed a lentiviral expression system that will enable the inducible expression of ADAMTS4 in primary cultured astrocytes, as well as inducible expression *in vivo*, in a rat model of spinal cord injury. The pLVX-Tet-On Advanced lentiviral vector constitutively expresses the tetracycline-controlled transactivator, rtTA-Advanced. The response lentiviral vector, pLVX-Tight-puro contains a polylinker site for insertion of the gene of interest, the expression of which is controlled by the P_{tight} promoter (a modified Tet-responsive element). Upon induction with doxycycline, rtTA-Advanced expressed from the pLVX-Tet-On lentiviral DNA binds to the *P*Tight promoter on the response vector, activating transcription of the downstream gene. The ADAMTS-4 insert in the 703-6 (ADAMTS-4 expression) plasmid was excised with EcoRI and XbaI, and was ligated into the pLVX-Tight-puro vector cut with the same enzymes. The ligation product was verified by restriction digestion and sequencing. The pLVX-Tet-On and the pLVX-Tight-puro-ADAMTS-4 plasmids were used to produce lentivirus with the Lenti-X HT packaging system in HEK293T cells. An additional vector was used to prepare a pLVX-tight-puro-luciferase lentivirus for optimizing transactivator and response vector ratios in astrocyte cell cultures. We have used the Lenti-XTM qRT-PCR Titration kit (Clontech) to titer our lentiviral preparations relative to a kit-provided standard having a known copy number. Currently, we have obtained lentivirus titers that are adequate to transduce primary astrocyte cultures. Experiments are in progress to optimize activator-response virus ratios and doxycyline concentations to activate gene expression. Experiments will be performed to quantify ADAMTS-4 expressed from the lentivirus in primary astrocytes. Proteoglycan degradation will be assessed by western blot analysis, as was done for cultures transfected with the ADAMTS-4 plasmid expression construct.

1c. Using the above system, we will determine if aggrecanase-mediated degradation of CSPGs in primary rat cortical astrocytes induces growth-inhibiting (CSPG-producing) astrocytes to become growth permissive (CSPG-degraded), and thereby foster regeneration of adult neurons (CST, RST, DC) (months 8-11).

We have learned from early stages of this study that neurite outgrowth inhibition is difficult to quantify using "choice" assays (proteoglycan stripe assays) previously used in our laboratory, since we are addressing large numbers of cells that need to be analyzed. However, such standard assays are useful and will continue to be used for analysis of *specific* growth cone responses to substratum proteoglycans, in smaller sample sizes. To address our need to analyze large numbers of neurons, we have optimized a commercially available assay for high-throughput neurite outgrowth analysis (Cellomics Neurite Outgrowth Kit) to measure the inhibitory effects of substratum bound proteoglycans on neurite outgrowth. In this assay, Neuroscreen^{TM}-1 (NS-1) cells, a derivative of the PC12 cell line, are plated in a 96-well plate, and each well is treated with increasing concentrations of NGF-1 to stimulate neurite outgrowth. To test the effect of CSPGs on outgrowth, plates are incubated with serial dilutions of proteoglycan prior to plating. Adsorbed PG is quantified by ELISA assay on duplicate plates. Following two days of outgrowth, cells are fixed and stained to reveal nuclei and neurites, which are detectible at different fluorescent wavelengths. A microscope with a motorized, computer-driven stage is used to obtain multiple images per well of nuclei and neurites. These images have been analyzed using the Neurite Tracer plugin with ImageJ software. Neurite outgrowth normalized to cell number was plotted vs. NGF-1 concentration for each concentration of proteoglycan adsorbed to the plate surface. In this manner, we have obtained curves to quantify neurite inhibition as a function of proteoglycan concentration **(Figure 6).** These assays are currently being conducted to quantify outgrowth inhibition potential of the different astrocyte PG pools described above.

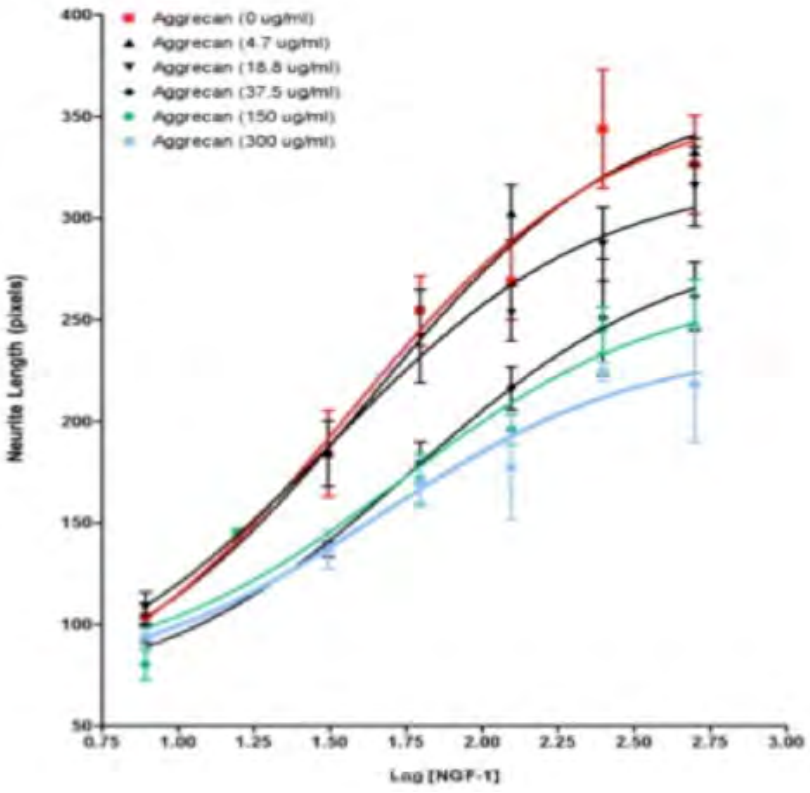

Figure 6. Neurite outgrowth assay. Graph shows increase in neurite outgrowth in response to NGF-1 in cultures without adsorbed aggrecan (red), and decreasing outgrowth relative to increasing concentrations of adsorbed aggrecan at the indicated concentrations.

1d. We will test the responses of other neurons, e.g. 5HT, which have been shown to be robust following SCI in previous studies (months 12-13).

Not yet addressed.

Using the assays above, we will determine CSPG production (ELISA), CSPG cleavage (Western blot analyses), and aggrecanase activity (enzyme activity assays and immunostaining for neoepitopes) (Miwa, Gerken et al. 2006; Miwa, Gerken et al. 2006). Further, we will isolate aggrecanase-generated fragments and test their effect(s) on elongating axons in vitro.

The recombinant ADAMTS-4 preparation described above will be used to produce PG fragments, which will be tested for outgrowth inhibition (or stimulation) activity.

Task 2. We will determine if aggrecanase-mediated degradation of CSPGs produced *in vivo*, in a rat spinal cord injury model system, will permit regeneration (months 13-36), and the effects of combining aggrecanase and chondroitinase treatments.

2a. Lentiviral transfection of ADAMTS-4 in an SCI model system will be performed, using currently approved methods (IACUC 0085M2005; appr. update July 2009 [Snow]; 01094M2006; appr date Sep 2009 [Smith]). We will transduce, using a lentiviral vector, ADAMTS-4 within an injured region of the rat spinal cord (dorsal hemisection), (months 13-18; to adapt the treatment to an established injury model system and develop successful surgical techniques).

Lentiviral preparation is described above for Task 1.

The focus of our SCI work *in vivo* is hand function. Thus, we have relied on our collaborator, Dr. Stephen Onifer, who uses and is optimizing for his own studies, a spinal cord injury at the C-4/5 level in rat. We began by using forceps and scissors to perform a dorsal hemisection, and examined loss of paw function (related to both sensory and motor deficits). While this method was reasonably successful, examination of tissue sections (histology; **Figure 7**) prepared from these animals showed variability in the lesion volume between animals. These animals sometimes did not fare well, having a motor deficit that is beyond the discreet loss of hand function we are attempting to obtain. Further, the injuries were not always consistent. Reproducible, consistent injuries and resulting consistent behavioral deficits are vital for proper determination of the success of aggrecanase/chondroitinase treatments.

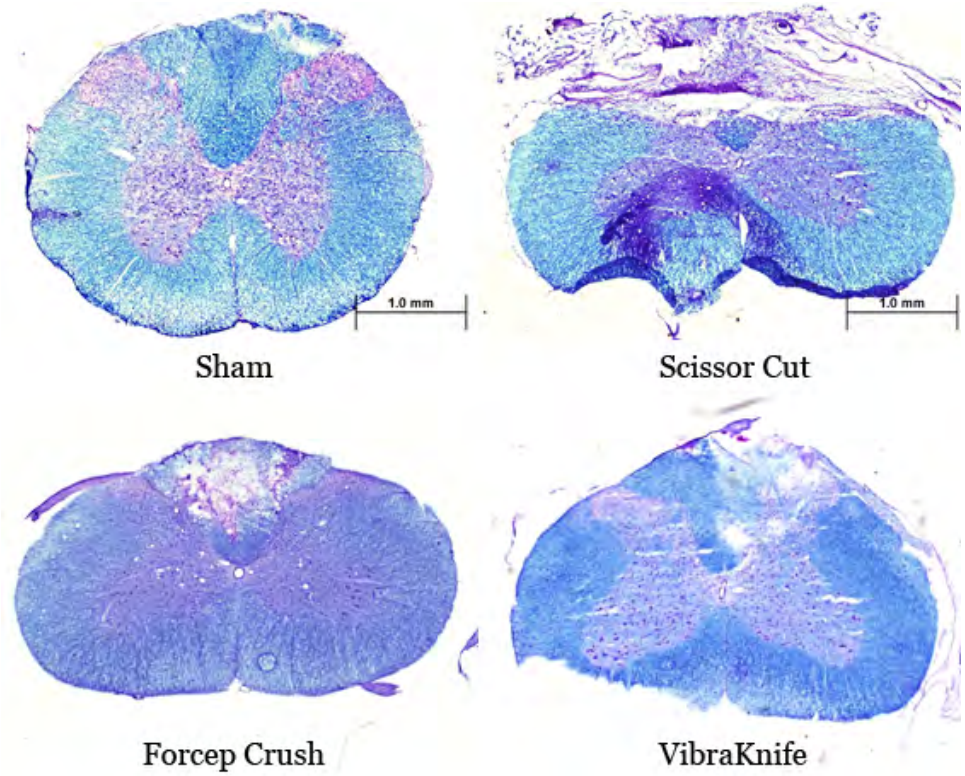

Figure 7. Transverse sections of rat spinal cord for 1) sham, 2) injured with scissors, 3) injured with forceps, and 4) injured using the Vibraknife, all at the cervical level (C3/4). Sections stained with cresyl violate and counterstained with Alcian Blue. The scissor cut gave the greatest anatomical deficit, as well as the most severe behavioral deficit. All but the Vibraknife injury resulted in wide variations in injury volume and behavior.

For this reason, we have moved to a new injury device, the Vibraknife **(Figures 7 and 8),** fabricated by colleagues at nearby University of Louisville. Dr. Onifer has used this device repeatedly and has had excellent reproducibility in his animals. The University of Louisville has fabricated a new machine for The University of KY. We have moved our animals from the BBSRB to the new BioPharm building and are creating a new surgical set up so that Dr. Onifer and I can easily share this piece of equipment. Under Dr. Onifer's animal protocol, we performed a pilot experiment using this device.

The procedure was as follows: Anesthetized adult male Sprague Dawley rats received an incision in the skin of the back of the shaved and cleansed neck. After muscle dissection, a laminectomy of the dorsal cervical (C5 and C6) vertebrae were performed. A transverse incision was made in the dura with the tip of a sterile 30-G needle between the C6 and C7 dorsal root entry zones. A dorsal-to-ventral laceration of the dorsal columns with a vibrating, custom-made razor blade attached to the LISA-Vibraknife was performed at the level of the dorsal corticospinal tracts. The muscle and skin incisions were closed. Fine forepaw digit use was expected to be mildly dysfunctional after the cervical hemisection lesion, based on previous studies (Onifer et al., 1997, 2005; Massey et al., 2006; Onifer et al., 2007; Titsworth et al., 2007; Massey et al., 2008). The remainder of forelimb, hindlimb, eating, drinking, respiration, bladder, bowel, and locomotor functions was normal after recovery from anesthesia. Therefore, no additional post-surgery or post-operation veterinary care was required beyond that which is routinely performed for spinal cord injured rats. The results of this pilot study are shown in **Figure 8**. Further, we are having great success in consistency of injury and of behavioral deficits following the injury (see below).

We will continue with this model for subsequent studies. We have modified our animal protocol (#2010-0702) to reflect this change in procedure. Following *in-vitro* characterization of our lentivirus in the coming year, we will begin experiments to infect spinal cord injury sites *in vivo* in dorsal hemisected rats, and will closely analyze ADAMTS-4 expression and aggrecan degradation using immunohistochemical techniques.

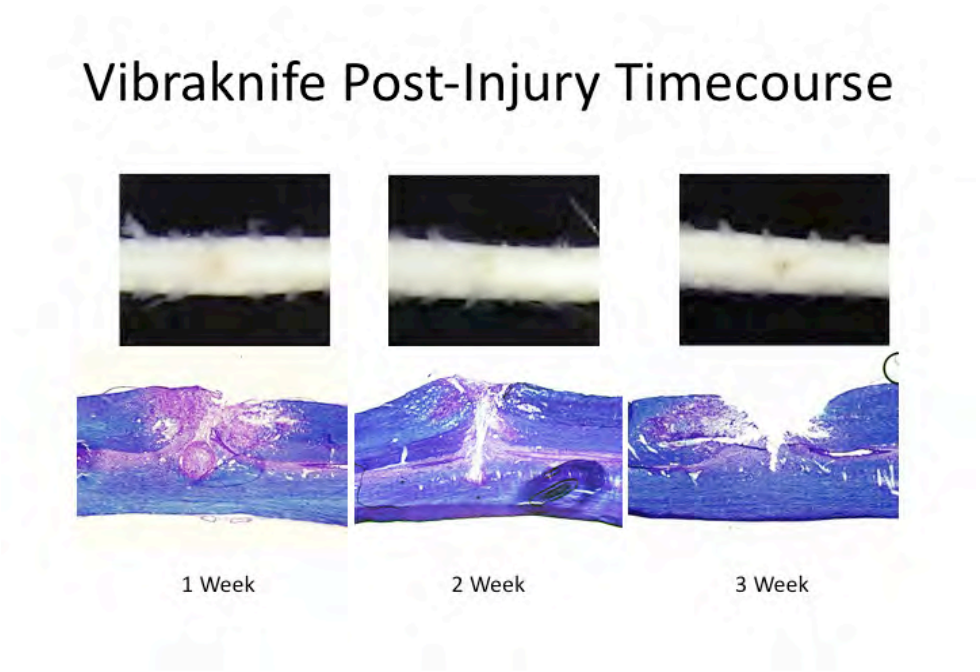

Figure 8. Post injury histology of Vibraknife-injured rat spinal cord at 1, 2 and 3 weeks post injury. Longitudinal sections taken at or near midline; n=6.

2b. Aggrecan degradation in the lesion will be monitored with anti-neoepitope antibodies that will recognize aggrecan fragments. Using a variety of microscopy methods and established tract tracing techniques, neurons traversing the glial scar depleted of aggrecan by ADAMTS-4 will be quantified relative to untreated rats. (months 19-24; to gather supportive information to validate results of 3a).

Not yet addressed.

2c. Histological assays to identify all cell types and molecules of interest *in vivo*. (months 25-28)

Some histological analysis has already been performed to identify the lesion volume in dorsal hemisected rats. Neurons and PGs have been labeled with neuron specific antibodies *in vitro*.

2d. Behavioral assays. Repeat *in vivo* paradigm and test behavioral recovery using the *Reach, Grasp and Pellet Retrieval* test (motor), the *Grid Walking* test (motor), and the *Sticker Attention* test (sensory) (months 29-36).

A technician and two undergraduate students have performed the Sticker Attention test on uninjured and dorsal hemisected rats, to obtain baseline data, and assess the value of the behavioral test. Preliminary data showed that injured rats required two times as long to notice and remove the small, plantar surface-adhered stickers than did uninjured rats **(Figure 9),** which was the expected trend. This assay will be used to test sensory deficit and recovery in aggrecanase and/or chondroitinase treated animals.

Our collaborator Dr. Stephen Onifer is optimizing the pellet retrieval test for motor function assessment, and we will use this technique in our next cohort of injured animals. A technician and two undergraduate students are optimizing this behavioral test currently, and it will soon be ready for use on our treated animals.

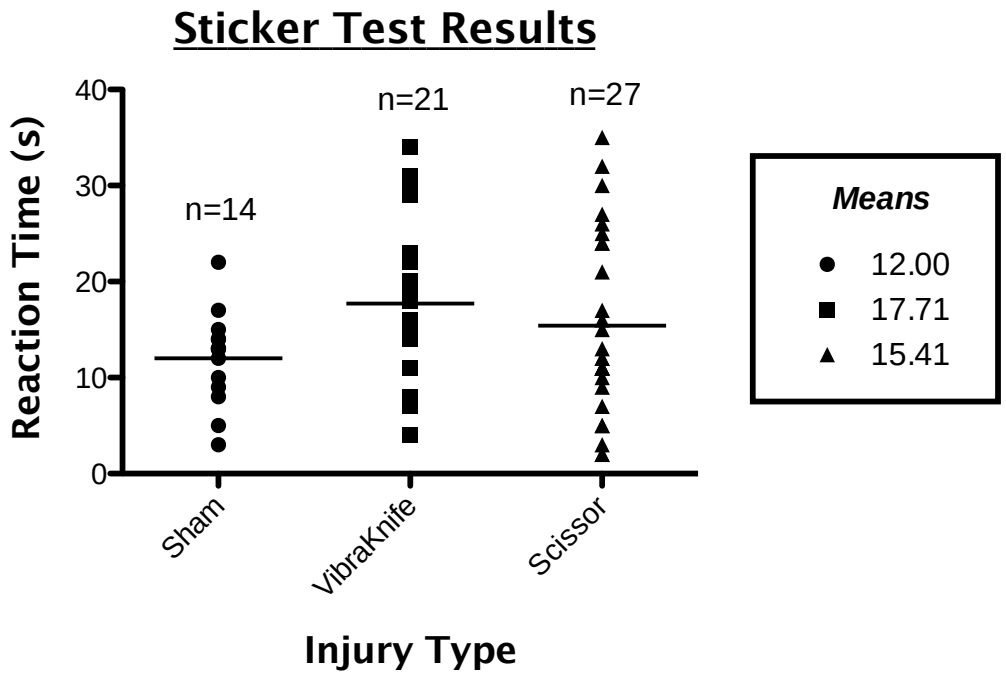

Figure 9. Spinal cord injured rats were tested 3 weeks following injury. A small sticker is placed on the plantar surface of the injured forepaw, and the animals are observed and timed to record the amount of time required to remove the sticker. The reaction time is measured in seconds. The above graph shows results for sham animals, in comparison to both scissor-injured and Vibraknife-injured animals (see Task 2 above). N= # of trials for sham (2 animals), Vibraknife (3 animals), and scissors (4 animals).

Summary

The above accomplishments represent significant advances in a one-year period and put us well on our way to completing the experiments described in our original grant application. In all, we are slightly ahead of our projected timeline.

Personnel: Dr. George Smith was originally included as a collaborator for the studies on this application, due to his lentiviral preparation of chondroitinase. Dr. Smith left The University of Kentucky in August 2011 for a position at Temple University in Philadelphia. Dr. Onifer will assume the role originally played by Dr. Smith, such that Dr. Smith's exit will not be disruptive to the progress of the proposed studies. Other than this personnel change, and the adoption of a modification of the injury method (Vibraknife), there are no other changes to the proposed studies

Key research accomplishments

The major accomplishments of the study to date are:

1) Purification and characterization of injured astrocyte proteoglycans

2) Generation of three ADAMTS-4 expression constructs

3) Production of recombinant ADAMTS-4 protein for experiments *in-vitro* and *in vivo*

4) Preparation of pLVX-Tet-On Advanced, and pLVX-Tight-Pur-ADAMTS-4 vectors

5) Optimization and use of a commercially available assay for high-throughput neurite outgrowth (Cellomics Neurite Outgrowth Kit) to measure the inhibitory effects of substratum bound proteoglycans on neurite outgrowth *in vitro*.

6) Dedveloped a reproducible injury model in which to test aggrecanase and chondroitinase

7) Performed and assessed Sticker Attention Test validity for assessment of aggrecanase and chondroitinase treatments

Reportable outcomes.

I. Abstracts *(complete abstracts in Appendix).*

TRANSIENT EXPRESSION AND PURIFICATION OF AGGRECANASE (ADAMTS-4) FROM HEK293T CELLS. Jonathan Davies, Diane M. Snow*, and T. M. Hering*. The University of Kentucky, Spinal Cord and Brain Injury Research Center, Lexington, KY 40536. *(this data will be presented by Mr. Davies at the International Symposium on Neural Regeneration, Dec. 7-11, 2011, Monterey, CA.; this conference does not normally accept undergraduates but accepted his abstract; this will be his first professional meeting).*

II. Summer Research Grants

Jonathan Davies, undergraduate summer research grant from the Appalachian & Minority Science, Technology, Engineering and Mathematics Majors (AMSTEMM) program. *(this grant allowed Mr. Davies to work 20 hr/wk on the project during the summer – June-Aug, 2011).*

III. Scholarships

Jonathan Davies, undergraduate scholarship from the Appalachian & Minority Science, Technology, Engineering and Mathematics Majors (AMSTEMM) program. *(this scholarship allowed Mr. Davies to build on the techniques he learned and the data he acquired during the summer of 2011; he is currently in my lab and plans to continue through summer 2012).*

Conclusions

Importance of implications of completed research: Thus far, our work has generated the specific tools and methodologies necessary to test aggrecanase and chondroitinase *in vivo* for recovery of hand function following SCI. We have about 6 months more work to do in the characterization phases of these tools and methods, including the validation of the new injury device. Thereafter, all experimentation will focus directly on treatment and assessments.

Recommended changes: No recommended changes beyond those already implemented (see Body).

"So what?" *(evaluate knowledge gained as a scientific or medical product*): No scientific or medical product is yet evident at this early stage of the project.

a. References

References from original application; still applicable. Not all references listed here are cited in progress report, but all in progress report are listed here.

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Appendices.

*Davies et al., abstract (Reportable Outcome)

*Snow, D. M. Biosketch

*Hering, H.M. Biosketch

TRANSIENT EXPRESSION AND PURIFICATION OF AGGRECANASE (ADAMTS-4) FROM HEK293T CELLS. Jonathan Davies, Diane M. Snow*, and T. M. Hering*. The University of Kentucky, Spinal Cord and Brain Injury Research Center, Lexington, KY 40536.

Following spinal cord injury (SCI), chondroitin sulfate proteoglycans (CSPGs, e.g. aggrecan), are up-regulated and constitute a major component of an inhibitory extracellular matrix (ECM). The influence of this complex barrier must be overcome to promote neuronal regeneration. The aggrecanase ADAMTS-4, a member of the *ADAMTS* (A Disintegrin And Metalloprotease with ThromboSpondin motifs) family of proteases, is capable of degrading CSPGs. Our hypothesis is: *ADAMTS-4 can effectively degrade CSPGs and thereby attenuate inhibition of sensory axons in a model of axon regeneration in vitro.* To address this hypothesis, we transfected Human Embryonic Kidney (HEK293T) cells with an ADAMTS-4-FLAG expression construct. The resulting aggrecanase, purified using FLAG-tag antibody affinity column chromatography, and analyzed using the Pierce BCA assay, SDS-PAGE, and Western blotting, will be used for activity assays *in vitro*. The results thus far show that ADAMTS-4 can be successfully produced by transfecting HEK293T with an expression vector. The expressed ADAMTS-4-FLAG fusion protein can be identified by its reactivity with antibodies to the FLAG epitope, and to the ADAMTS-4 protein itself. ADAMTS-4 was retained in the cell layer, and was not in the media. Once purification is complete, enzymatic activity will be assessed by the enzyme's ability to degrade purified aggrecan *in vitro*, and outgrowth assays on degraded aggrecan *in vitro* will be undertaken. Alone or in combination with other CSPG degradative proteins, ADAMTS-4 could become a useful therapy to selectively degrade CSPGs that inhibit neuronal outgrowth thereby promoting regeneration and recovery of function in SCI patients. [*Support: NIH (R01 NS053470), the Department of Defense (W81XWH-10-1-0778; the UK Office of Undergraduate Research, and AMSTEMM*]. * These authors contributed equally to this work.

Principal Investigator/Program Director (Last, First, Middle): **Snow, Diane M.**

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Diane M. Snow, PhD	Professor with Tenure
eRA COMMONS USER NAME	
Diane.Snow	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The University of Akron, Akron, OH	B.S.	1982	Biology/German
The University of Akron and NEOUCOM	M.S.	1985	Neuroscience
Case Western Reserve University, Cleveland, OH	Ph.D.	1989	Neuroscience
Case Western Reserve University, Cleveland, OH	PostDoc	1990	Neuroscience

Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

1984-1986	Technician, Cleveland Clinic Foundation, Dept. of Brain and Vascular Research
1986-1990	Brumagin Fellowship for Spinal Cord Injury Research
1991-1994	NIH National Research Service Award, F32
1992-1993	Macalester College, Minneapolis, MN, Faculty
1994-1996	Research Assistant Professor, The University of Minnesota, Dept. of Cell Biology and Neuroanatomy, Minneapolis, MN
1996-2002	**Assistant Professor**, Dept. of Anatomy and Neurobiology, The Univ. of Kentucky, Lexington, KY
2002-2008	**Associate Professor with Tenure**, Dept. of Anatomy and Neurobiology, The Univ. of Kentucky, Lexington
2003-04	Wethington Research Award
2001-2007	**Faculty Associate, Spinal Cord and Brain Injury Research Center**, Univ. of KY, Lexington, KY
2007	National ACE Network Leadership Award (Advancement of Women in Higher Education) for "an outstanding, innovative, and visionary leadership program – *Circles of Power*"
2008	Kentucky Academy of Sciences - *Superlative Award, Distinguished University Scientist*
2008-pres	**Professor and Endowed Chair**, Spinal Cord and Brain Injury Research Center (SCoBIRC); Dept of Anat and Neurobiology, The University of Kentucky, Lexington, KY
2007-11	Wethington Research Award
2008; 09	Abraham Flexner Master Educator Award *(2011 pending)*
2009	Society for the Promotion of Undergraduate Research (SPUR) - *Excellent Undergraduate Research Mentor Award*
2010	Sarah Bennett Holmes Award, contribution to development of women professionals, *Women's Forum*
2011-12	Southeast Conference Academic Consortium – Administrative Leadership Development Program Fellow
2011-pres	Board of Directors and Advisory Board member, Exomedicine Institute, NASA-funded zero-gravity medical research

A. Peer-reviewed publications (*selected of 50***).**

Snow, D.M., D.A. Steindler, and J. Silver (1990) Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. *Dev. Biol*. 138:359-376.

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Wilson, M.T. and **Snow, D.M**. (2000) Chondroitin sulfate proteoglycan expression pattern in hippocampal development: potential regulation of axon tract formation. *J. Comp. Neurol*.424:532-546.

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Snow, D. M.*,* Smith, J.D., and*.* Gurwell, J. A. (2002), Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. *J. Neurobiol* 51:285-301*.*

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Johnson, W.E., Caterson,B, Eisenstein, S.M., Hynds, D.L., ***Snow, D. M., and *Roberts, S. (*shared senior authorship)**. (2002) Human intervertebral disc aggrecan inhibits nerve growth in vitro. *Arthritis and Rheumatism* 46(10):2658-2664.

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Zhang, G. ,R.-L. Lin, M. Wiggers, D.M. Snow, and L.-Y. Lee. (2008) Altered expression of TRPV1 and sensitivity to capsaicin in pulmonary myelinated afferents following chronic airway inflammation in the rat. J. Physiol. (Lond) 586:5771-86. (PMID: 18832423)

Lin S, Liu M, Son YJ, Timothy Himes B, **Snow DM**, Yu W, Baas PW (2011) Inhibition of Kinesin-5, a microtubule-based motor protein, as a strategy for enhancing regeneration of adult axons. *Traffic* 12(3):269-86.

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B. **Research Support.** *On-going or completed within past 5 years:*

"Recombinant aggrecan variants having specifically modified CS chains and their regulation of axonal regeneration" - ACTIVE
P.I. D. Snow, 10% effort; 1/15/11-1/14/14
Agency: Kentucky Spinal Cord and Head Injury Research Trust

Aims: 1) To use shRNA to knock down specific biosynthetic enzymes in the CS synthesis pathway to produce modified CS chains on recombinant aggrecan (a CSPG). These specifically modified CS chains will be analyzed to confirm the nature of the predicted modification; and, 2) To determine the ability of CSPGs engineered in Aim 1 to inhibit neurite outgrowth.

"A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration" - ACTIVE
P.I. D. Snow, 20% effort; 9/30/10-9/29/13
Agency: Dept of Defense - CDMRP
Aims: 1) To determine if aggrecanase and chondroitinase-mediated degradation of CSPGs, either purified, or produced by primary rat cortical astrocytes, will foster neurite outgrowth in vitro; and 2) To determine if aggrecanase and chondroitinase-mediated degradation of CSPGs in a rat spinal cord injury model in vivo, will result in improved axonal regeneration and recovery of function.

"Designer PGs for CNS Injury" - ACTIVE
P.I. D. Snow, 30% effort; 9/1/07-8/31/12
Agency: NIH, NINDS; 1R01NS053470-01A2
Aims: Identification and manipulation of inhibitory microdomains of glial scar chondroitin sulfate proteoglycans in vitro focusing on cortical astrocytes interactions with sensory neurons.

"Role of TRPV1 in Airway Hypersensitivity Induced by Inflammation" - ACTIVE
PI: Lu-Yuan Lee; Co-I: D. Snow, 5% effort; 9/1/09 - 8/31/11
Agency: NIH - National Heart, Lung and Blood Institute; 1R01 (HL096914-01)
Aims: To investigate the role of TRPV1 in the development of airway hypersensitivity when chronic airway inflammation is induced by allergen sensitization.

"Designer PGs for Spinal Cord Injury"
PI - D. Snow; 20% effort; 11-1-07 to 10-31-010
Agency: Christopher and Dana Reeve Foundation
Aims: identification and manipulation of inhibitory microdomains of glial scar neurocan in vitro and in vivo using shRNA technology.

"PGs, Monastrol, and Regeneration"
P.I.s - D. M. Snow and P. Baas, 12-31-08 to 12-30-09
Agency: Christopher and Dana Reeve Foundation
Aims: To determine if the kinesin-5 inhibitor, monastrol, attenuates CSPG-induced axonal inhibition.

"Engineering Chondroitinase AC for Expression Within the Injured Spinal Cord"
P.I. G. Smith, Co-I: D. Snow, 5% effort; 1-15-07 to 1-14-09**.**
Agency: Kentucky Spinal Cord and Head Injury Research Trust
Aims: To engineer mammalian chondroitinase via lentiviral technology to facilitate regeneration.

"Cell-permeant peptides for modulating neuronal growth cone behavior"
PI: M. Wilson; Snow 10% effort; 1-1-07 to 9-1-07
Agency: NIH; SBIR; R43-NS051878
Aims: To develop cell-permeant peptides to manipulate neuronal cytoskeletal elements and promote neurite outgrowth.

"Mammalian-produced chondroitinase AC mitigates axon inhibition by chondroitin sulfate proteoglycans"
P.I. G. Curinga (Snow, Faculty Sponsor and Mentor), 7/1/05-6/30/07.
Agency: University of Kentucky Postdoctoral Fellowship for Women in Under-Represented Areas,
Aims: To engineer astrocyte-produced chondroitinase AC to alleviate axonal inhibition in vitro and in vivo.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Hering, Thomas M.	Research Associate Professor
eRA COMMONS USER NAME THERING	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Kent State University, Kent, OH	B.S.	1974	Biology
Cleveland State University, Cleveland, OH	M.S.	1977	Biology
Case Western Reserve Univ., Cleveland, OH	Ph.D.	1985	Experimental Pathology
Rush University, Chicago, IL	postdoc	1985	Biochemistry/Molecular Biology
University of Washington, Seattle, WA	postdoc	1987	Biochemistry/Molecular Biology

A. Personal Statement

In September 2010 I joined the Department of Anatomy and Neurobiology/Spinal Cord and Brain Injury Research Center at the University of Kentucky to become a full time member of the faculty. I have collaborated with Dr. Diane Snow for several years prior to my arrival at UK, while at Case Western Reserve University and during my Visiting Professorship at Washington University in St. Louis. My research specialization is in the biochemistry and molecular biology of the extracellular matrix (ECM), with a current emphasis on the role of ECM components in neuronal regeneration. The role of the ECM in neuronal regeneration is only beginning to be understood. My previous experience has been in the area of musculoskeletal-related connective tissue research, an area of study in which the biochemistry of proteoglycans and their metabolic and catabolic pathways is fairly advanced. It is my intention to bring biochemical and molecular biological approaches for the analysis of ECM to the study of CNS repair mechanisms.

B. Positions And Honors

Positions And Employment

1974-75 Teaching Assistant, Dept. of Biology, Cleveland State University, Cleveland, OH
1975-78 Research Assistant, Dept. of Pediatrics, Case Western Reserve University, Cleveland, OH
1985-87 Postdoctoral Fellow and Instructor, Depts. of Biochemistry and Orthopaedics Rush University, Chicago, IL
1987-88 Postdoctoral Research Associate, Dept. of Orthopaedics, University of Washington, Seattle, WA
1988-89 Research Associate, Dept. of Orthopaedics, University of Washington, Seattle, WA
1989-97 Assistant Professor, Dept. of Medicine, Div. of Rheumatic Diseases Case Western Reserve University, Cleveland, OH
1991-97 Assistant Professor, Dept. of Anatomy, Case Western Reserve University, Cleveland, OH
1997-98 Associate Professor, Dept. of Medicine, Div. of Rheumatic Diseases, Case Western Reserve University, Cleveland, OH
1997-2009 Associate Professor, Dept. of Anatomy, Case Western Reserve University, Cleveland, OH
1998-2009 Associate Professor, Dept. of Orthopaedics, Case Western Reserve University, Cleveland, OH
2009 Adjunct Associate Professor, Dept. of Biomedical Engineering, Case Western Reserve University, Cleveland, OH.
2009-10 Visiting Associate Professor, Dept. of Orthopaedic Surgery, Washington University, St. Louis, MO
2010 Research Associate Professor, Department of Anatomy and Neurobiology/Spinal Cord and Brain Injury Research Center (SCoBIRC), University of Kentucky, Lexington, KY.

Professional Memberships

1985 Member, American Association for the Advancement of Science
1988 Member, American Society for Cell Biology
1989 Member, Orthopaedic Research Society
2002 Member, American Society for Matrix Biology
2004 Member, Osteoarthritis Research Society International

Honors And Awards

1979-82 USPHS Predoctoral Traineeship in Cellular and Molecular Biology, CWRU, Cleveland, OH
1985 USPHS Postdoctoral Traineeship in Orthopaedics, Rush Medical College, Chicago, IL
1987-88 NIH Individual Postdoctoral Fellowship.
1991-96 NIH/AR40829 (FIRST Award),
1994 National Service Citation, Arthritis Foundation,
1990 Invited Presentation,Gordon Conference on Proteoglycans, Andover, NH
1995 Elected to Medical and Scientific Committee, Arthritis Foundation, Northeast Ohio Chapter,.
2008 Invited Presentation, Gordon Conference on Proteoglycans, Andover, NH
2009-10 Osteoarthritis Research Society International (OARSI) Scholarship Award (Visiting Professorship at Washington University, St. Louis, MO.)

C. Selected Peer-Reviewed Publications (Selected from 27 peer-reviewed publications)

Most relevant to the current application

1. Miwa, H.E., Gerken, T.A., Huynh, T.D., Flory, DM., and Hering, T.M.: Mammalian expression of full-length bovine aggrecan and link protein: Formation of recombinant proteoglycan aggregates and analysis of proteolytic cleavage by ADAMTS-4 and MMP-13, Biochim. Biophys. Acta *1760*. 472-486, 2006.
2. Miwa, H.E., Gerken, T.A., and Hering, T.M.: Effects of covalently attached chondroitin sulfate on aggrecan cleavage by ADAMTS-4 and MMP-13 Matrix Biology, 25, 534-545, 2006.
3. Miwa, H. E., Duesler, L., Kazmi, N.H., Huynh, T.D., and Hering, T.M.: Conserved sequence in the aggrecan interglobular domain modulates cleavage by ADAMTS-4, Biochim. Biophys. Acta, 1790, 161-172, 2009.

Additional publications of importance to the field (in chronological order)

1. Hering, T.M., Marchant, R.E., and Anderson, J.M.: Type V collagen during granulation tissue development. Exp. Mol. Pathol., 39: 219-229, 1983
2. Hering, T.M., Suzuki, Y., and Anderson, J.M.: Collagen type distribution in healing of synthetic vascular prostheses. Conn. Tiss. Res., 15: 141-154, 1986.
3. Hering, T.M., and Sandell, L.J.: Biosynthesis and cell-free translation of swarm rat chondrosarcoma and bovine cartilage link proteins. J. Biol. Chem., *263*, 1030-1036,1988.
4. Hering, T.M., and Sandell, L.J.: Biosynthesis and processing of bovine cartilage link proteins, J. Biol. Chem., *265*, 2375-2382, 1990
5. Hering, T.M., Kollar, J., Huynh, T.D., Varelas, J.B., and Sandell, L.J.: Modulation of extracellular matrix gene expression in bovine high density chondrocyte cultures by ascorbic acid and enzymatic resuspension. Arch. Biochem. Biophys. *314*, 90-98 1994
6. Hering, T.M., Kollar, J. , Huynh, T.D. and Sandell, L.J.: Bovine chondrocyte link protein cDNA sequence: interspecies conservation of primary structure and mRNA untranslated regions, Comp. Biochem. Physiol. 112B(2), 197-203, 1995
7. Hering, T.M., Kollar, J., Huynh, T.D. and Varelas, J.B.: Prokaryotic expression, refolding, purification and functional characterization of the decorin as a maltose binding protein fusion. Anal. Biochem., *240*, 98-108, 1996
8. Hering, T.M., Huynh, T.D., Kollar, J., and Huang, A.: Complete coding sequence of bovine aggrecan: Comparative structural analysis. Archives Biochem. Biophys., *345*, 259-270, 1997
9. Johnstone, B., Hering, T.M. Caplan, A.J.., and Yoo, J.U.: In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells, Exptl. Cell Res., *238*, 265-272, 1998

10. Jabbour, L., Welter, J.F., Kollar, J. and Hering, T.M.: Sequence, gene structure, and expression pattern of *CTNNBL1*, a minor-class intron containing gene: Evidence for a role in apoptosis, Genomics, 81(3), 292-303, 2003
11. Hering, T.M., Kazmi, N.H., Huynh, T.D., Kollar, J., Xu, L. Hunyady, A.B., and Johnstone, B.: Characterization and Chondrocyte Differentiation Stage-Specific Expression of KRAB Zinc Finger Gene ZNF470, Exp. Cell. Res. *299*, 137-4, 2004.
12. McAlinden, A., Johnstone, B., Kollar, J., Kazmi, N., and Hering, T.M.: Expression of two novel alternatively spliced COL2A1 isoforms during chondrocyte differentiation, Matrix Biol. 27, 254-266, 2008.

D. Research Support

Ongoing Research Support

R01 NS053470-01A1 Snow (PI) 08/01/07-07/31/12
NIH/NINDS
Designer PGs for Spinal Cord Injury
The major goals of this project are to identify and manipulate chondroitin sulfate proteoglycan motifs to promote neural plasticity and regeneration.

Rehabilitation Research and Development Service Grant No. F6398R (Kath Bogie) 01/01/10-12/31/12
Department of Veterans Affairs
Determination of Physiological Mechanisms for Electrical Wound Therapy
The major goal of this project is to determine the effect of electrical fields upon wound healing

3048107568 Snow (PI) 09/30/10-09/29/13
US Army Medical Research and Materiel Command
A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration
The major goal of this project is to promote neuronal regeneration following spinal cord injury using aggrecanases (ADAMTS-4 or ADAMTS-5) to degrade inhibitory proteoglycans.

Grant #10-11A (Snow (PI) 01/01/11-12/31/12
Kentucky Spinal Cord and Head Injury Research Trust (KSCHIRT)
Recombinant aggrecan variants having specifically modified CS chains and their
regulation of axonal regeneration
The major goal of this project is to determine whether specific patterns of sulfation on chondroitin sulfate proteoglycans influence neuronal outgrowth.

Completed Research Support

NIH 1R21RR025397-01A1 (Hering/McAlinden) 07/01/09 - 06/30/11
NIH/NCRR
Novel Collagen II Alternative Transcripts and Mouse Skeletal Development
The goal of this project is to investigate the role of a newly discovered type II procollagen mRNA transcript, termed IIC, with respect to skeletal development.

R21 HD049834-01 Hering (PI) 4/01/06-3/31/09
NIH/NICHHD
Zfp28 and Mesenchymal Stem Cell Differentiation
The major goals of this project are to determine the role of Zfp28 in chondrogenic differentiation of mesenchymal stem cells by producing a conditional knockout mouse, and to identify genes regulated by Zfp28 using coupled chromatin immunoprecipitation and promoter microarray analysis.

NIH 1R13AR057640-01 (Hering/Chubinskaya) 07/01/09 – 06/30/10
NIH/NIAMS
Midwest Connective Tissue Workshop
This is an application for support of the 37th Midwest Connective Tissue Workshop to be held in Chicago, on May 8-9, 2009. The main goal of this conference is to bring together a diverse group of scientists and clinicians from the Midwest United States to promote and facilitate the progress of basic and translational research in the broad area of “connective tissue” and musculoskeletal disorders.